TOXICITY OF RED AND VIOLET DYES IN M18 GRENADES: MUTAGENIC SCREENING OF THREE DYES FOR MARKER GRENADES IN THE SALMONELLA REVERSION ASSAY AND THE L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA ASSAY

Final Report

Prepared by
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Three dyes (Solvent Red 1, Disperse Blue 3, and Disperse Red 11) were tested for mutagenicity in the Salmonella reversion assay and the L5178Y/TK$^{+/-}$ mouse lymphoma assay. These in vitro assays were performed both with and without exogenous activation provided by Aroclor induced rat liver S9. In the salmonella assay, Solvent Red 1 was positive with S9 activation in strain TA100 and negative to questionably positive in the other strains and activation conditions. Disperse Blue 3 was positive with S9 activation in strain TA1537 and negative to questionably positive under the other test conditions. Disperse Red 11 was positive with S9 activation in strain TA102, but negative to weakly positive with the other strains and activation conditions. Both Disperse Blue 3 and Disperse Red 11 were positive both with and without S9 activation in the mouse lymphoma assay. Solvent Red 1 could not be tested without activation. With S9 activation, it was weakly positive.

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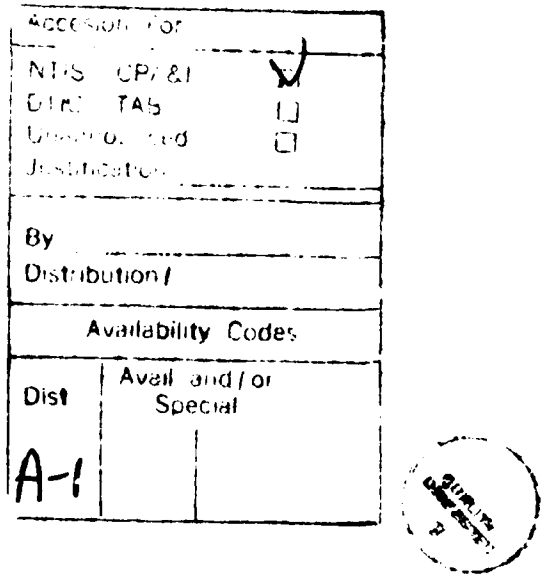
FOREWORD

All of the mutagenicity assays were performed in the Genetic Toxicology Division of the Health Effects Research Laboratory (HERL), U.S. Environmental Protection Agency (U.S. EPA), Research Triangle Park, NC. The Salmonella reversion assays were performed under the direction of Dr. Larry Claxton. The L5178Y/TK$^{+/-}$ mouse lymphoma assays were performed under the direction of Dr. Martha Moore. The experiments were performed by the U.S. EPA and Environmental Health Research and Testing (EHRT) personnel working within the EPA laboratory. EHRT provides research support to EPA under contract.



EXECUTIVE SUMMARY

Dyes are used by the military in M18 marker signaling grenades. A number of organic dyes are presently being evaluated for potential use in these grenades. In addition to engineering studies for their performance in the field, the U.S. Army is concerned with evaluating any potential health hazards that might result from personal contact with the dyes in the industrial setting. A part of this testing is the analysis of potential genetic toxicity.

Three dyes (Solvent Red 1, Disperse Blue 3, and Disperse Red 11) were tested for mutagenicity in the Salmonella reversion assay and the L5178Y/TK$^{+/-}$ mouse lymphoma assay. These in vitro assays were performed both with and without exogenous activation provided by Aroclor-induced rat liver S9. In the Salmonella assay, Solvent Red 1 was positive with S9 activation in strain TA100 and negative to questionably positive in the other strains and activation conditions. Disperse Blue 3 was positive with S9 activation in strain TA1537 and negative to questionably positive under the other test conditions. Disperse Red 11 was positive with S9 activation in strain TA102, but negative to weakly positive with the other strains and activation conditions. Both Disperse Blue 3 and Disperse Red 11 were positive both with and without S9 activation in the mouse lymphoma assay. Disperse Red 1 could not be tested without activation. With S9 activation, it was weakly positive.

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INTRODUCTION

Dyes are used by the military in M18 marker signaling grenades. A number of organic dyes are presently being evaluated for potential use in these grenades. In addition to engineering studies for their performance in the field, the U.S. Army is concerned with evaluating any potential health hazards that might result from personal contact with the dyes in the industrial setting. A part of this testing is the analysis of potential genetic toxicity.

Three dyes, Solvent Red 1, Disperse Blue 3, and Disperse Red 11 were tested for mutagenicity in the Salmonella reversion assay and the L5178Y/TK$^{+/-}$ mouse lymphoma assay. All three dyes were tested both with and without exogenous activation. In addition to testing in the "standard" Salmonella assay, all three dyes were also evaluated in the thin layer chromatography (TLC) modification.

MATERIALS AND METHODS

The dyes tested were: Solvent Red 1, Disperse Blue 3, and Disperse Red 11. Samples of these three dyes were supplied by the Army.

SALMONELLA REVERSION ASSAY

The procedures used were those of Maron and Ames (1983) with minor modifications. The guidelines of Claxton et al. (1987) were followed for both test performance and interpretation. Modifications are included in the description that follows. For each sample, the following six histidine-requiring strains were used: TA98, TA100, TA102, TA104, TA1537, and TA1538. The mechanisms by which each of these strains revert to prototrophy are fully discussed in other publications (Ames et al., 1975; Maron and Ames, 1983). In addition to these basic mechanisms, the reader should keep in mind the following salient points. These strains carry an rfa mutation which produces a deficiency in bacterial cell wall lipopolysaccharides and increases the cell's permeability to large molecules; the uvrB mutation decreases genetic repair; and the R-factor plasmid in strains TA98 and TA100 increases the spontaneous mutation rate. The six strains differ in the number of spontaneous revertants per plate generally found. Compounds which are known mutagens for the different strains, with and without activation, were included in each assay as positive controls. The retention of phenotypic characteristics was checked with each test by examining for histidine auxotrophy (lack of growth on histidine deficient medium), deep rough character (sensitivity to crystal violet on a disk), UV-repair deficiency (sensitivity to UV light), and the presence of the appropriate plasmid (resistance to ampicillin on a disk).

Preparation of Rat Liver S9 Mix

Male CD-1 (Fisher derived) rats weighing approximately 200 g were given a single intraperitoneal injection of Aroclor 1254 in corn oil (200 mg/ml) at a dose of 50 mg/kg of body weight. One day prior to termination the animals were taken off food but provided water ad libitum. The livers were aseptically removed and washed in sterile cold 0.15 M KCl. All subsequent steps were performed at 0° to 4°C with cold sterile solutions and sterile glassware. The livers were minced with scissors in 0.15 M KCl (3 ml/g wet weight liver) and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 9000 x g, the supernatant (S9) decanted and stored in convenient aliquots at -80°C.

The S9 was mixed with a cofactor solution containing 8 mmol $MgCl_2$. 32 mmol KCl, 5 mmol glucose-6-phosphate, and 4 mmol nicotinamide adenine dinucleotide in 100 mmol of sodium phosphate buffer, pH 7.4. The final S9 mix contained between 0.05 and 0.1 ml S9/ml cofactor solution.

Test Procedure

For revertant selection, minimal Vogel-Bonner medium E supplemented with 1.5% Difco Bacto agar and 2% glucose was used for base agar layers. The top agar (0.6% Difco Bacto agar, 0.5% NaCl) was supplemented with minimal amounts of histidine and biotin. The bacterial broth culture ($1\text{-}2 \times 10^9$ viable cells per ml) and the test material dissolved in dimethylsulfoxide (DMSO) (supplied sterile, spectrophotometric grade) were added to the top agar. For tests without activation, 0.5 ml of cofactor solution was added instead of the S9 mix to the top agar. The plates were incubated in the dark at 37°C for 72 hr. The plates were examined for background growth, and the number of colonies per plate were counted using an Artek 880 automatic colony counter.

Statistical Analyses

Statistical tests and computer programs used were those of Stead et al. (1981). This model assumes that revertant colony formation at any dose follows a Poisson process, while the mean number of revertants per plate is a nonlinear function of up to four parameters. The resultant system of nonlinear equations is solved using a modified Gauss-Newton iterative scheme to obtain maximum likelihood estimates of the model parameters. Significance of the key parameters was tested by fitting reduced models and using likelihood ratio tests.

The determination of positives was based on the following criteria:

- The data must not vary significantly from a Poisson distribution ($p > 0.01$).
- The data must be acceptable by the test of adequacy of fit of the model ($p > 0.01$).
- The test for mutagenicity (the slope of the curve) must be significant ($p < 0.01$).
- All positive and negative controls must have given expected responses as compared to HERL, U.S. EPA historical values and those published by Ames et al. (1975).
- Histidine cross-feeding and/or contamination must not have been shown to occur.

The modeling of the bioassay provides a valuable aid to the researcher; however, each curve was (and needs to be) examined individually to assure confidence in the apparent conclusions of the statistical process. For example, if the dose-response data "fit" statistically a horizontal line (response vs. dose), the model will under some circumstances record a mutagenicity p-value less than 0.01; however, since the slope equals zero the response is negative.

An equivocal response occurs when (1) test results were not reproducible, (2) a low-level but no dose-related increase in his colonies is obtained, or (3) when an increase was observed at only a single dose level. See the papers

by Ames et al., 1975; Claxton et al., 1987; and Maron and Ames, 1983 for further discussions of test result interpretation.

The reader must also keep in mind that these particular tests were performed to maximize the chance of detecting a mutagenic response and not to provide comparative slope values. Examination of the data also shows that test doses were often adjusted due to results of a previous test. These adjustments obviously can shift results from a negative response to positive result (e.g. if a compound was initially tested at too low a dose- response range) and may alter the slope value (e.g. providing more doses in the central portion of the dose-response curve).

The minimum testing requirements were as follows:

- A minimum of five doses at half-log intervals with the highest dose being highly toxic, as shown by background clearing and/or reduction in expected revertant counts per plate.

- Spontaneous and positive controls done at least in duplicate and providing the expected response as compared to HERL, U.S. EPA historical values and those published by Ames et al. (1975).

- Positive controls (in duplicate) for the microsomal activation combination used are within normal ranges as compared to HERL, U.S. EPA historical values and those published by Ames et al. (1975).

- These minimum criteria are carefully explained in other publications (Ames et al., 1975; Claxton et al., 1987).

TLC/SALMONELLA ASSAY

Because the mutagenicity of technical-grade chemicals might arise from the presence of a contaminant, it is useful to have a rapid screening system to detect this possibility. A rapid, inexpensive system that was employed for this purpose is the TLC/Salmonella mutagenicity assay. With this system (Bjorseth et al., 1982), the sample is chromatographed on TLC plates, and the components for the Salmonella mutagenicity assay are applied directly to the developed chromatogram. The subsequent appearance of localized clusters of mutant colonies suggests the presence of a mutagenic constituent. This assay has been successfully applied to a number of different substances (Bjorseth et al., 1982; Moller et al., 1983; Houk and Claxton, 1986). The modification used for this study (Houk and Claxton, 1986) uses a single agar overlay poured over the TLC plate. This overlay contains the tester strain of bacteria and any exogenous activation system that is used.

Test Procedure and Analysis

Commercially available glass-backed silica or cellulose high performance thin layer chromatography (HPTLC) plates (10 cm x 10 cm) are developed (without sample application) in absolute ethanol. After development they are dried at room temperature under the hood for 5 min, and then dried an additional 20 min at 80°C. Plates are subsequently treated as sterile.

Samples are then spotted on the plate via a micropipette in varying volumes, depending on their mutagenic potency. When possible, all dyes were spotted at concentrations ranging from 2 μg to 1 mg per spot. Samples are evenly spaced along the width of the plate 1.5 cm from the bottom; ordinarily four sample volumes are applied across the plate. Plates are then lowered into a developing tank containing the appropriate solvent system at a depth of 1 cm.

Chloroform is the first solvent applied. If chloroform does not separate the components of the sample, various other systems are tried. Samples can also be applied singly to a plate at a point 1.5 cm from the bottom and 1.5 cm from the left-hand side and run 2-dimensionally (with two different solvent systems) to further separate the constituent parts. In most instances, chloroform has proven sufficient.

The mobile phase is allowed to migrate up the plate at room temperature until the solvent front is about 1/2 cm from the top of the plate. Plates are then removed from the chamber and allowed to air dry under the hood.

At this point, plates are examined under UV light for fluorescent activity. Components may also be made visible on parallel plates by conventional techniques or examined under ambient light for the presence of additional patterns.

In order for the TLC plates to fit into the 150x150 mm disposable petri dishes, the upper two edges must be removed with the aid of a glass cutter. Plates are placed into the dishes with the developed side facing up. To 30 ml of VBME agar at 45°C are added 500 μl suspension ($1x10^9$ bacteria/ml) of the tester strain and 1.5 ml of S9 mix, when required. Contents are slowly poured into the space between the TLC plate and the edge of the petri dish, and the dish rotated for even distribution. The agar is allowed to harden, and the plates inverted and incubated at 37°C for 72 hours.

Plates are then examined for clusters of colonies, toxic zones, and/or total revertant count. If colonies are evenly spread across the TLC plate (for example, as seen with highly polar compounds), a total increase in mutant colonies is seen. In most cases, the increase in mutant colony numbers will be associated with the mutagenic constituent that has migrated during chromatography. Toxic components are characterized by a zone devoid of bacterial colonies.

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA ASSAY

Solvent Red 1, Disperse Blue 3, and Disperse Red 11 were evaluated for mutagenicity in the L5178Y/TK$^{+/-}$ mouse lymphoma assay using the procedures described by Turner et al. (1984). This in vitro mammalian system evaluates mutations affecting the thymidine kinase locus. This assay may be particularly useful in a test battery since the mutants quantitated can be divided, by colony size, into two distinct groups (small-colony and large-colony mutants). These two classes of mutants appear to reflect the relative clastogenic and mutagenic potential of the compound tested. Hozier et al.

(1981, 1983) have shown that the majority of small-colony mutants reflect chromosome damage affecting chromosome 11 (the location of the thymidine kinase gene), while large-colony mutants appear to represent small-scale, perhaps single-gene damage.

Cell Line and Cell Maintenance

The $TK^{+/-}$ -3.7.2C heterozygote of L5178Y mouse lymphoma cells (supplied by Dr. Donald Clive) was utilized. This cell line was routinely grown in supplemented Fischer's Medium for Leukemic Cells of Mice (see below). Cells were monitored daily (except for weekends) for acceptable growth rates. For weekends, the cells were sufficiently diluted so that they would remain in log-phase growth; weekend cell doubling times were always determined. Weekly, prior to use in the assay, cells were cleansed of spontaneous $TK^{-/-}$ cells by 24-hr growth in the presence of thymidine (3 mg/ml), hypoxanthine (5 mg/ml), methotrexate (0.1 mg/ml), and glycine (7.5 mg/ml) (THMG). This was followed by 24-hr growth in THG (THMG minus methotrexate) medium. Stock cells were stored in liquid nitrogen.

Media

$TK^{+/-}$ -3.7.2C cells were cultivated in Fischer's Medium for Leukemic Cells of Mice supplemented with 31 mg/ml penicillin (1650 units/mg), 50 mg/ml streptomycin sulfate, 0.1% Pluronic F68, 0.22 mg/ml sodium pyruvate (F_0P), and 10% horse serum to make $F_{10}P$. Medium was heat inactivated at 55°C for 45 minutes. Cells were cloned in the above described supplemented medium to solidify the cloning medium for colony formation. The selective agent used for mutation at the TK locus was 1 μg/ml trifluorothymidine (TFT).

Preparation of Chemical Solutions

Concentrations were prepared on a weight per volume basis. DMSO was used as the solvent. A fresh stock of test material was used for each separate experiment.

Preparation of the Metabolic Activation System

Aroclor 1254-induced rat liver S9 was purchased from Sitek, Inc. who prepared it in the following manner: Rats weighing 200-300 g were injected intraperitoneally with a 2:1 mixture of Aroclor 1254 in corn oil (500 mg of total Aroclor/kg body weight). After 5 days the animals were sacrificed by CO_2 exclusion of air. They were totally immersed in a solution of Wescodyne for approximately 3 seconds and their heads quickly excised. The livers were removed and placed in preweighed beakers containing 0.25 M sucrose. Livers were washed three times in 50-100 ml portions of cold 0.25 M surcose to yield 3 ml per gram of liver. Livers were minced and then homogenized in a blender for two 15-second periods. The homogenate was centrifuged at 9000 x g for 10 min at 4°C. The lipid layer was removed and discarded. The supernatant was pooled and aliquoted into sterile serum vials and placed directly into liquid nitrogen vapor phase containers for storage prior to shipping. A sterility check and activity test for standard promutagens in the mouse lymphoma assay were performed prior to shipping.

Upon receipt the S9 was stored at -70°C in a Revco freezer and tested for the ability to activate benzo(a)pyrene to mutagenic metabolites as based on induced mutant frequency in the standard mouse lymphoma assay.

The S9 mix was made in a 1:4 ratio of S9 to cofactor mix. Cofactor mix was made just prior to addition of S9 and consisted of F_0P (Fischer's Medium supplemented but without horse serum), 8 mg/ml B-nicotinamide adenine dinucleotide phosphate (NADP), and 15 mg/ml DL-isocitric acid trisodium salt (isocitrate). This solution was filter sterilized, mixed with freshly thawed S9, and kept on ice until used.

Mutagenicity Assay

The doses chosen for the mutagenicity assay were based on the results of a dose-ranging study. One 50-ml Corning polypropylene tube seeded with $6x10^6$ cells in 6.0 ml of medium with a reduced amount of serum (5% instead of 10%) was used for each dose. Four ml of serum-free Fischer's medium (F_0P) were added to each tube. The compound was dissolved in DMSO at 100 times the highest concentration to be tested. Sufficient solvent was added to each tube so that after addition of the test compound all tubes contained the same final solvent concentration. Normally 1% DMSO is the maximum used in this assay to deliver the test compound. The test compound was added to each appropriately labelled tube, the tubes were then regassed with 5% CO_2-in-air and incubated in a roller drum at 37°C for 4 hr. Following the 4-hr exposure period, the tubes were centrifuged for 10 min at 200 x g and the supernatant containing the test compound was discarded. The cells were then washed twice in 10 ml of $F_{10}P$ (2 x 10 min centrifugations at 200 x g), and resuspended in 20 ml of fresh $F_{10}P$ to a final cell concentration of $3x10^5$ cells/ml. The tubes were regassed with 5% CO_2-in-air and incubated in the roller drum at 37°C.

Positive control compounds were tested with each experiment. Ethyl methanesulfonate (EMS, 400 μg/ml) was used without exogenous activation, and benzo(a)pyrene (BAP, 3 and 4 μg/ml) with S9 activation. Cell counts were determined with a Coulter Counter Model ZBI at 24 and 48 hr after exposure to the compound. Each culture was diluted daily to $2x10^5$ cells/ml. At the end of 48 hr, the cells were cloned. Cloning allows for the selective growth and enumeration of mutant cells in a soft agar cloning medium (CM) and for the determination of cloning efficiency. Fifteen ml of each culture were spun in a centrifuge at 200 x g for 10 min and the supernatant decanted. Approximately 1-2 ml of $F_{10}P$ were added to each culture for resuspension of the cell pellet. The cell pellet was vigorously resuspended to ensure a single cell suspension and placed in 100 ml of CM to give a cell concentration of $3x10^4$ cells/ml. The flasks were labelled with the appropriate culture number and selective agent to be used (TFT). The cells were allowed to acclimate for 30 min and then a 1:50 dilution was made. (One ml was transferred from each culture to prelabelled flasks containing 50 ml of CM.) After mixing for 15 min, 1.0 ml from each 50-ml flask was transferred to 100 ml of CM and labelled with the culture number and viable count (VC) (cell concentration = 6 cells/ml). The selective agent, 1 mg/ml TFT, was added to the flasks containing $3x10^4$ cells/ml. Three petri plates per TFT and VC flask were poured, 33 ml per 100 mm petri plate. The plates were chilled at -20°C for 12 min, placed in a 5% CO_2 incubator, and incubated for 10-12 days at 37°C.

At the end of the incubation period the plates were scored for the number of colonies per plate using an Artek colony counter model 880. TFT- resistant colonies from selected cultures showing positive mutagenicity were sized by differential counts at periodic size discriminator settings. This information was expressed as histograms showing the relative proportions of small- and large-colony TFT-resistant mutants. This approach is a possible means of characterizing the type of mutagenic events occurring [i.e. single gene mutations (large colonies) or chromosomal aberrations affecting the TK and other genes (small colonies)].

Calculation of Mutant Frequency

The mutant frequency was calculated by dividing the total number of mutant colonies for each culture by the number of viable cells plated for the culture (as determined by the VC plates). The spontaneous mutant frequency (solvent control) was subtracted from the total mutant frequency to give the induced mutant frequency.

Criteria for the Evaluation of the Results

The following criterion (based on the statistical methods of Clive et al., 1979) must be met to designate the test compound as a definite positive: One or more doses (from at least two separate assays) must show a significant increase in mutant frequency at reasonable (>10%) survival and there must be a multi-point dose-related response at adequate (>10% survival) cytotoxicities.

If there is no significant increase of the mutant frequency over background and if the compound has been adequately tested (with and without metabolic activation, reasonably spaced doses, adequate cytotoxicity--sufficient doses in the 10-20% survival range), then the results will be interpreted as negative.

The minimum criteria for an acceptable assay are: (1) plating efficiency of the solvent control is between 50 and 115%, (2) spontaneous mutant frequency of the solvent control is less than 100×10^6 and (3) positive controls show a definite positive response.

RESULTS AND DISCUSSION

SALMONELLA REVERSION ASSAY

The Salmonella bioassay is frequently used to screen substances for genotoxicity including potential carcinogenicity. The three dyes were tested in the standard plate incorporation assay using six strains supplied by Dr. Bruce Ames. The six strains used were TA98, TA100, TA102, TA104, TA1537, and TA1538. In addition, the three dyes were also tested using the TLC/Salmonella assay. A summary of the results is presented in Tables 1, 2, 3, and 4. The results are very heterogeneous.

For all three dyes, three of the strains, namely TA98, TA104, and TA1538, gave no positive responses either with or without exogenous metabolic activation. TA104 and TA1538, however, did provide a questionable response for Disperse Blue when exogenous activation was used. In addition, TA104 with exogenous activation gave a questionable response for Solvent Red 1. These questionable responses could not be resolved due to the dense coloration of the plates (which created enumeration difficulties and obscured precipitation when it occurred), the potential overlapping of toxicity and mutagenicity, and the limits of solubility of the dye. TA1537 gave a clearly positive response for the Disperse Blue dye when activation was used; however, TA1537 without activation did not give a definitive positive response. TA102 appeared to

TABLE 1. SUMMARY RESULTS OF ARMY DYES TESTED IN THE SALMONELLA TYPHIMURIUM MUTAGENICITY ASSAY*

Test Condition	Exog. Act.	Solvent Red 1	Disperse Blue 3	Disperse Red 11
TA100	+	positive	negative	negative
TA100	-	negative	negative	negative
TA1538	+	negative	equivocal	negative
TA1538	-	negative	negative	negative
TA98	+	negative	negative	negative
TA98	-	negative	negative	negative
TA1537	+	negative	positive	negative
TA1537	-	negative	equivocal	negative
TA102	+	equivocal	weak pos.	positive
TA102	-	negative	equivocal	weak pos.
TA104	+	equivocal	equivocal	negative
TA104	-	negative	negative	negative

* See text and publications by Ames et al., 1975; Maron and Ames, 1983; and Claxton et al., 1987 for explanations on the determination of positive, negative, and equivocal responses. A "weak pos." means a weak positive and shows that no consistent response above twofold the spontaneous count was seen.

TABLE 2. SALMONELLA TYPHIMURIUM MUTAGENICITY TEST RESULTS FOR SOLVENT RED 1*

DOSE µg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA100 MARCH 22, 1988			
POS	408	23	1132	25
0.0	132	21	150	5
0.3	139	1	195	15
3.0	192	4	142	3
30.0	231	8	152	10
300.0	195	15	129	9
	TA100 APRIL 01, 1988			
POS	576	36	470	2
0.0	121	11	119	22
0.3	120	16	138	10
3.0	159	19	141	11
10.0	193	11	141	14
30.0	196	15	133	14
100.0	192	21	132	13
	TA100 APRIL 11, 1988			
POS	588	14	682	17
0.0	108	8	122	12
0.3	116	1	118	9
3.0	143	7	126	8
10.0	184	17	136	8
30.0	222	16	122	10
100.0	175	10	131	3
	TA102 APRIL 22, 1988			
POS	1418	427	1086	48
0.0	391	2	297	1
0.3	419	22	296	12
3.0	424	6	327	15
30.0	441	17	265	15
300.0	355	13	243	25

TABLE 2. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
		TA102 MAY 2, 1988		
POS	1219	21	1163	38
0.0	397	16	316	30
0.3	411	43	266	3
3.0	402	15	256	15
10.0	464	16	270	24
30.0	473	25	299	22
100.0	431	131	252	3
		TA102 MAY 6, 1988		
POS	1227	41	905	33
0.0	386	16	260	18
0.3	493	17	285	9
3.0	470	12	274	17
10.0	498	1	285	23
30.0	473	8	272	14
100.0	506	15	289	21
		TA102 JULY 15, 1988		
POS	949	72		
0.0	234	22		
50.0	242	19		
100.0	244	47		
200.0	167	14		
300.0	203	17		
		TA104 APRIL 22, 1988		
POS	681	7	536	33
0.0	337	34	263	4
0.3	379	4	349	103
3.0	871	567	1844	477
30.0	446	40	271	24
300.0	343	18	207	58
		TA104 MAY 2, 1988		
POS	732	31	484	14
0.0	287	24	261	12
0.3	280	16	259	3
3.0	339	18	248	14
10.0	337	22	233	17
30.0	361	14	268	14
100.0	350	30	233	23

TABLE 2. (Continued)

DOSE µg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
		TA104 MAY 6, 1988		
POS	781	23	595	14
0.0	341	30	265	3
0.3	309	12	243	4
3.0	368	21	238	12
10.0	420	30	236	20
30.0	452	13	265	14
100.0	379	23	252	19
		TA1537 APRIL 22, 1988		
POS	194	57	205	72
0.0	16	1	7	2
0.3	15	1	8	0
3.0	14	0	7	1
30.0	15	3	6	1
300.0	9	1	10	1
		TA1537 MAY 6, 1988		
POS	292	20	254	93
0.0	15	3	7	1
0.3	18	2	9	4
3.0	19	6	9	7
10.0	14	2	7	2
30.0	23	5	11	5
100.0	14	6	7	1
		TA1538 MARCH 22, 1988		
POS			329	17
0.0			13	4
0.3			15	0
3.0			10	4
30.0			7	1
300.0			13	1
		TA1538 APRIL 01, 1988		
POS	263	44	398	21
0.0	32	7	17	11
0.3	33	7	14	3
3.0	26	7	18	1
10.0	26	3	19	9
30.0	32	1	18	5
100.0	26	4	21	3

TABLE 2. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA1538 APRIL 11, 1988			
POS	235	16	339	13
0.0	23	7	13	5
0.3	27	6	12	3
3.0	34	10	12	6
10.0	22	5	10	3
30.0	30	8	13	1
100.0	23	2	13	4
	TA98 MARCH 22, 1988			
POS	335	30	207	23
0.0	40	1	36	10
0.3	37	4	17	5
3.0	52	3	29	8
30.0	34	4	19	3
300.0	42	6	26	6
	TA98 APRIL 1, 1988			
POS	456	3	248	17
0.0	40	3	24	5
0.3	56	6	29	3
3.0	53	9	41	10
10.0	50	8	33	3
30.0	55	6	36	13
100.0	44	3	33	5
	TA98 APRIL 11, 1988			
POS	360	7	181	37
0.0	27	7	19	6
0.3	37	5	11	6
3.0	36	6	19	3
10.0	30	2	22	4
30.0	41	1	18	2
100.0	39	6	19	7

* POS: Positive control compounds, which are: Sodium azide (3.0 μg, TA100 and TA1535, -S9), 2-nitrofluorene (3.0 μg, TA98 and TA1538, -S9), 9-aminoacridine (100 μg, TA1537, -S9), 2-aminoanthracene (0.5 μg, TA100 TA98, and TA1538, +S9) (3.0 μg, TA104 and TA1537, +S9), dihydroxyanthraquinone (30.0 μg, TA102, +S9), and methylglyoxal (50 μg, TA104, -S9).

TABLE 3. SALMONELLA TYPHIMURIUM MUTAGENICITY TEST RESULTS FOR DISPERSE BLUE 3*

DOSE	WITH ACTIVATION		WITHOUT ACTIVATION	
µg/plate	MEAN	SD	MEAN	SD
	TA100 MARCH 22, 1988			
POS	408	23	1132	25
0.0	132	21	150	5
3.0	140	3	149	4
30.0	155	5	143	8
300.0	116	2	135	15
3000.0	195	15	129	8
	TA100 APRIL 01, 1988			
POS	576	36	470	2
0.0	121	11	119	22
3.0	137	9	133	4
10.0	162	6	134	11
30.0	140	6	134	3
100.0	123	18	141	3
300.0	109	14	128	15
	TA100 APRIL 11, 1988			
POS	588	14	682	17
0.0	108	8	122	12
3.0	130	9	124	14
10.0	118	24	126	11
30.0	114	12	120	10
100.0	121	25	146	8
300.0	64	6	114	7
	TA102 APRIL 22, 1988			
POS	1418	427	1086	48
0.0	391	2	297	1
3.0	487	38	319	8
30.0	706	8	346	15
300.0	558	54	326	25
3000.0	141	9	102	8

TABLE 3. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
		TA102 MAY 2, 1988		
POS	1219	21	1163	38
0.0	397	16	316	30
3.0	298	16	289	16
10.0	345	21	270	24
30.0	387	27	299	22
100.0	418	32	252	3
300.0	380	43	316	30
		TA102 MAY 6, 1988		
POS	1227	41	905	33
0.0	386	16	260	18
3.0	471	9	310	26
10.0	568	36	340	22
30.0	685	2	356	19
100.0	837	20	402	13
300.0	569	57	387	48
		TA102 JULY 15, 1988		
POS	876	51		
0.0	142	19		
25.0	239	14		
50.0	234	17		
100.0	247	17		
200.0	287	15		
		TA104 APRIL 22, 1988		
POS	681	7	536	33
0.0	337	34	263	4
3.0	386	0	271	21
30.0	976	239	261	34
300.0	645	458	232	0
3000.0	124	11	183	46
		TA104 MAY 2, 1988		
POS	732	31	484	14
0.0	287	24	261	12
3.0	280	17	222	9
10.0	305	4	253	24
30.0	261	12	241	21
100.0	259	68	256	6
300.0	100	2	208	23

TABLE 3. (Continued)

DOSE µg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
		TA104 MAY 6, 1988		
POS	781	23	595	14
0.0	341	30	265	3
3.0	329	10	256	17
10.0	325	22	256	21
30.0	377	27	238	14
100.0	313	10	248	19
300.0	138	10	200	18
		TA1537 APRIL 22, 1988		
POS	194	57	205	72
0.0	16	1	7	2
3.0	17	4	10	5
30.0	32	2	9	0
300.0	65	3	9	1
3000.0	65	14	25	4
		TA1537 MAY 2, 1988		
POS	254	24	116	22
0.0	15	3	9	5
3.0	25	6	7	4
10.0	44	5	11	3
30.0	49	10	13	4
100.0	102	7	14	3
300.0	123	14	18	3
		TA1537 MAY 6, 1988		
POS	292	20	254	93
0.0	15	3	7	1
3.0	24	7	9	5
10.0	19	2	11	4
30.0	29	4	13	4
100.0	43	2	9	4
300.0	46	11	17	8
		TA1538 MARCH 22, 1988		
POS			329	17
0.0			13	4
3.0			15	1
30.0			14	2
300.0			7	2
3000.0			11	2

TABLE 3. (Continued)

DOSE	WITH ACTIVATION		WITHOUT ACTIVATION	
µg/plate	MEAN	SD	MEAN	SD
		TA1538 APRIL 01, 1988		
POS	263	44	398	21
0.0	32	7	17	11
3.0	37	4	15	0
10.0	49	5	20	4
30.0	65	7	22	7
100.0	60	10	12	3
300.0	43	2	13	2
		TA1538 APRIL 11, 1988		
POS	235	16	339	13
0.0	23	7	13	5
3.0	32	5	12	3
10.0	59	5	14	9
30.0	72	4	13	4
100.0	66	7	17	1
300.0	46	4	13	5
		TA1538 JUNE 24, 1988		
POS	713	25		
0.0	25	4		
0.5	26	4		
1.0	23	6		
3.0	31	1		
5.0	32	6		
10.0	33	3		
		TA98 MARCH 22, 1988		
POS	335	30	207	23
0.0	40	1	36	10
3.0	46	12	22	60
30.0	99	10	23	5
300.0	65	11	18	5
3000.0	28	7	15	1

TABLE 3. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA98 APRIL 1, 1988			
POS	456	3	248	17
0.0	40	3	24	5
3.0	60	3	33	60
10.0	97	11	30	4
30.0	115	8	30	4
100.0	98	8	32	5
300.0	77	17	29	2
	TA98 APRIL 11, 1988			
POS	360	7	181	37
0.0	27	7	19	6
3.0	45	4	15	4
10.0	72	4	20	5
30.0	80	7	26	2
100.0	90	18	20	7
300.0	57	4	18	4

* POS: Positive control compounds, which are: Sodium azide (3.0 μg, TA100 and TA1535, -S9), 2-nitrofluorene (3.0 μg, TA98 and TA1538, -S9), 9-aminoacridine (100 μg, TA1537, -S9), 2-aminoanthracene (0.5 μg, TA100, TA98, and TA1538, +S9) (3.0 μg, TA104 and TA1537, +S9), dihydroxyanthraquinone (30.0 μg, TA102, +S9), and methylglyoxal (50 μg, TA104, -S9).

TABLE 4. SALMONELLA TYPHIMURIUM MUTAGENICITY TEST RESULTS FOR DISPERSE RED 11*

DOSE	WITH ACTIVATION		WITHOUT ACTIVATION	
µg/plate	MEAN	SD	MEAN	SD
	TA100 MARCH 22, 1988			
POS	408	23	1132	25
0.0	132	21	150	5
7.5	158	12	128	14
75.0	159	6	149	11
750.0	112	1	122	9
7500.0	88	9	77	19
	TA100 APRIL 01, 1988			
POS	576	36	470	2
0.0	121	11	119	22
0.75	138	2	154	10
7.5	130	2	129	3
75.0	123	6	115	14
250.0	137	30	122	11
750.0	112	20	95	2
	TA100 APRIL 11, 1988			
POS	588	14	682	17
0.0	108	8	122	12
0.75	118	11	133	8
7.5	130	10	113	15
75.0	127	8	109	20
250.0	140	15	109	8
750.0	98	11	87	11
	TA102 APRIL 22, 1988			
POS	1418	427	1086	48
0.0	391	2	297	1
7.5	525	29	334	11
75.0	577	30	385	16
750.0	474	230	298	26
7500.0	224	180	129	32

TABLE 4. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
		TA102 MAY 2, 1988		
POS	1219	21	1163	38
0.0	397	16	316	30
0.75	384	11	302	26
7.5	535	11	373	25
75.0	748	76	501	19
250.0	627	84	493	48
750.0	484	25	370	91
		TA102 MAY 6, 1988		
POS	1227	41	905	33
0.0	386	16	260	18
0.75	410	11	283	23
7.5	499	2	327	11
75.0	624	22	377	12
250.0	619	31	360	2
750.0	319	55	345	37
		TA104 APRIL 22, 1988		
POS	681	7	536	33
0.0	337	34	263	4
7.5	339	33	287	4
75.0	382	27	271	30
750.0	315	14	215	9
7500.0	135	18	119	19
		TA104 MAY 2, 1988		
POS	732	31	484	14
0.0	287	24	261	12
0.75	279	18	226	7
7.5	318	15	233	14
75.0	265	12	219	8
250.0	209	17	151	20
750.0	209	16	164	21
		TA104 MAY 6, 1988		
POS	781	23	595	14
0.0	341	3C	265	3
0.75	294	9	246	11
7.5	311	29	247	10
75.0	302	28	180	6
250.0	341	25	182	6
750.0	299	8	163	23

TABLE 4. (Continued)

DOSE µg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA1537 APRIL 22, 1988			
POS	194	57	205	72
0.0	16	1	7	2
7.5	9	4	9	5
75.0	27	8	6	4
750.0	18	4	8	3
7500.0	14	3	10	1
	TA1537 MAY 2, 1988			
POS	254	24	116	22
0.0	15	3	9	5
0.75	15	0	11	5
7.5	18	2	12	7
75.0	10	5	10	5
250.0	19	4	10	3
750.0	22	6	14	5
	TA1537 MAY 6, 1988			
POS	292	20	254	93
0.0	15	3	7	1
0.75	13	6	7	3
7.5	18	10	13	5
75.0	15	10	11	5
250.0	16	1	10	1
750.0	15	4	14	6
	TA1538 MARCH 22, 1988			
POS			329	17
0.0			13	4
7.5			12	5
75.0			10	5
750.0			9	5
7500.0			4	3
	TA1538 APRIL 01, 1988			
POS	263	44	398	21
0.0	32	7	17	11
0.75	29	3	11	3
7.5	36	3	10	4
75.0	44	5	16	5
250.0	36	12	12	5
750.0	39	8	12	2

TABLE 4. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA1538 APRIL 11, 1988			
POS	235	16	339	13
0.0	23	7	13	5
0.75	90	34	12	4
7.5	48	5	8	3
75.0	40	4	20	6
250.0	48	17	12	4
750.0	49	11	14	8
	TA1538 AUGUST 19, 1988			
POS	287	24		
0.0	26	7		
0.5	24	3		
1.0	31	7		
3.0	24	5		
5.0	22	3		
10.0	34	2		
	TA98 MARCH 22, 1988			
POS	335	30	207	23
0.0	40	1	36	10
7.5	66	11	25	7
75.0	81	11	20	5
750.0	57	2	19	3
7500.0	16	2	13	4
	TA98 APRIL 1, 1988			
POS	456	3	248	17
0.0	40	3	24	5
0.75	55	6	32	6
7.5	77	11	33	5
75.0	111	30	32	4
250.0	91	5	36	5
750.0	76	10	25	4

TABLE 4. (Continued)

DOSE μg/plate	WITH ACTIVATION MEAN	SD	WITHOUT ACTIVATION MEAN	SD
	TA98 APRIL 11, 1988			
POS	360	7	181	37
0.0	27	7	19	6
0.75	37	7	20	1
7.5	57	8	25	8
75.0	65	6	17	3
250.0	86	4	22	1
750.0	80	18	18	1

* POS: Positive control compounds, which are: Sodium azide (3.0 ug, TA100 and TA1535, -S9), 2-nitrofluorene (3.0 μg, TA98 and TA1538, -S9), 9-aminoacridine (100 μg, TA1537, -S9), 2-aminoanthracene (0.5 μg, TA100, TA98, and TA1538, +S9) (3.0 μg, TA104 and TA1537, +S9), dihydroxyanthraquinone (30.0 μg, TA102, +S9), and methylglyoxal (50 μg, TA104, -S9).

respond to all three dyes; however, the response of TA102 to Solvent Red 1 and Disperse Blue could only be classified as either a questionable or weak positive. The lack of clarity in TA102 results was due to the aforementioned difficulties in testing these dyes and to the high spontaneous counts typically seen with this tester strain. Solvent Red 1 was positive in TA100 when exogenous metabolic activation was used.

TLC/SALMONELLA ASSAY

The TLC/Salmonella procedure was used to confirm the plate incorporation protocol results, to examine for the presence of significant amounts of contaminants, and to determine (if contaminants were present) whether or not any mutagenicity was associated with the minor constituents. Except for Disperse Blue, the dyes chromatographed with starting spot concentrations ranging from 2 μg per spot to 1 mg per spot. Due to solubility problems, the highest concentration of Disperse Blue that could be applied was 600 μg. Tests were done with Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537. Testing was independent of the plate incorporation tests that were done with the same dyes. A summary of results is given in Table 5.

TABLE 5. QUALITATIVE SUMMARY OF THE TLC/SALMONELLA ASSAY RESULTS FOR THREE ARMY DYES

Condition Examined	Solvent Red 1	Disperse Blue 3	Disperse Red 11
Chromatography			
a. White light	red; single band	blue; two bands	purple
b. UV light	bright orange; single band	none	pink-orange; extra band at origin
Mutagenicity			
TA100+	Negative	Negative	Negative
TA100-	Negative	Negative	Negative
TA98+	Negative	? Negative	Negative
TA98-	Negative	Negative	Negative
TA1537+	Negative	Positive	Negative
TA1537-	Negative	Positive	Negative
TA1535+	Negative	Negative	Negative
TA1535-	Negative	Negative	Negative

Solvent Red 1

The single band resulting from this chromatographed dye was a bright red color under normal light. When examined under UV light, these streaks (which migrated with the solvent front) fluoresced a bright orange. This dye was negative in all four strains both with and without exogenous activation. At the higher doses, slight toxicity was observed when this dye was tested without activation.

Disperse Blue 3

At the lowest concentrations, only a single blue band was apparent on the developed TLC plate. At higher concentrations, two separate blue bands became apparent at the solvent front. At the highest concentrations, the sample streaked from the origin to the solvent front. This dye was not mutagenic in strains TA100 and TA1535 either with or without activation. Using TA1537, the dye was mutagenic both with and without S9. Without S9 activation, the dye demonstrated more than a twofold increase in the average number of revertants per plate. At the lower doses a slight cluster effect was seen; however, the cluster effect was more evident at higher doses. For an explanation of how to interpret this qualitative test, see the section on Test Procedure and Analysis and the paper by Houk and Claxton, 1986. With S9, the response was even more pronounced. This dye gave a slightly elevated increase (of approximately 40%) in the average number of TA98 revertant colonies per plate; however, with S9, no cluster effect was seen.

Disperse Red 11

Under normal light the visible bands and streaks were a purple color. Under long-wave UV light, the streaks fluoresced a pink-orange color; however, this fluorescent color was less apparent at concentrations above 200 μg due to the sample being such a deep purple. The developed bands were approximately 5 mm behind the solvent front. Also, at the point of origin, a fluorescent pink-orange circle with a dark purple leading segment was seen. This dye appeared negative in the four strains used both with and without exogenous metabolic activation.

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA ASSAY

In experiment 1 of the Solvent Red 1 series (Table 6), cells were treated with the chemical in the absence of S9 activation up to 10 μg/ml. Ideally in an experiment, the chemical will induce either a mutant frequency $\geq$ 2X that of the negative controls or a toxic response in which one or more dosed points has a relative total growth between 10 and 20%. Because no positive responses were obtained and dosed points outgrew the negative controls, experiment 1 resulted in a "no test."

The maximum solubility of Solvent Red 1 was determined to be 3 mg/ml in DMSO. In experiment 2 (Table 6), cells were dosed up to 60 μg/ml. All points tested were delivered in 200 μl DMSO, and no precipitate was observed. The highest doses did not induce sufficient cytotoxicity, and the trial was

TABLE 6. MOUSE LYMPHOMA ASSAY OF SOLVENT RED 1 WITHOUT METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($x10^6$)
Experiment 1						
Neg. Control	100	507	185	100	100	73
Neg. Control	100	525	146	100	100	56
Pos. Control (EMS 400 μg/ml)	61	336	1850	65	40	1101
1	109	581	755	113	123	60
5	100	595	152	115	115	51
10	115	458	178	89	102	78
Experiment 2						
Neg. Control	100	447	155	100	100	69
Pos. Control (EMS 400 μg/ml)	64	288	1848	61	39	1283
30	89	387	115	87	77	59
40	90	408	187	91	82	92
50	90	497	162	111	100	65
60	87	525	187	117	103	71

determined to be a "no test." Due to the lack of cytotoxicity at the maximum level of solubility, Solvent Red 1 could not be successfully evaluated without activation.

S9 activation elevated the induced mutant frequency and the cytotoxicity of the Solvent Red 1 (Table 7). Although no positive points occurred in the first experiment, an increase in mutant frequency appeared at doses greater than 6.1 μg/ml. In the second experiment, the chemical was weakly positive (slightly greater than 2X background) at doses of 8.7 μg/ml and higher. In the third experiment, all doses of 7.5 μg/ml or higher induced a weakly positive mutagenic response. It should be noted that concentrations used in these experiments were very close together and the resultant dose-response curve shows a plateau response. When the TFT-resistant mutants were analyzed for the induction of small- and large-colony mutants (Figure 1), it was clear that neither type of mutant predominated.

Cells treated with Disperse Blue 3 exhibited a positive response without exogenous activation (Table 8) in two separate experiments. The chemical went into solution in DMSO, but formed a separate, lower layer when added to media. After vortexing, the chemical was soluble in the media. All centrifuge tubes

TABLE 7. MOUSE LYMPHOMA ASSAY OF SOLVENT RED 1 WITH METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($\times 10^6$)
Experiment 1						
Neg. Control	100	511	188	100	100	74
Neg. Control	100	506	181	100	100	72
Pos. Control						
(BAP 3 μg/ml)	72	307	756	80	57	372
(BAP 4 μg/ml)	35	269	1079	53	18	802
4.0	90	546	230	107	97	84
5.0	80	533	248	105	84	93
5.3	79	479	239	94	74	100
5.6	71	469	223	92	66	95
5.8	69	504	213	99	68	85
6.1	65	482	305	95	62	127
6.5	62	498	250	98	61	100
6.75	29	479	286	94	28	119
7.0	58	478	276	94	54	115
7.5	42	453	274	81	37	121
8.0	30	432	281	85	26	130
Experiment 2						
Neg. Control	100	499	193	100	100	77
Neg. Control	100	548	210	100	100	77
Pos. Control						
(BAP 3 μg/ml)	84	505	792	96	81	314
(BAP 4 μg/ml)	55	397	1091	76	41	560
5.7	83	562	244	107	89	87
6.1	81	520	272	99	81	105
6.5	72	544	264	104	75	97
6.75	73	508	282	97	71	111
7.0	71	584	263	112	79	90
7.3	58	527	289	101	58	110
7.5	57	538	348	103	59	129
8.0	46	510	338	97	45	132
8.2	31	496	395	100	31	152
8.5	32	565	380	108	34	134
8.7	28	497	511	95	27	206
8.9	25	456	404	87	22	177
9.0	16	459	468	88	14	204

TABLE 7. Continued

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($x10^6$)
Experiment 3						
Neg. Control	100	500	228	100	100	91
Neg. Control	100	469	190	100	100	81
Pos. Control						
(BAP 3 μg/ml)	72	337	847	70	50	503
(BAP 4 μg/ml)	30	272	1059	56	17	779
5.3	73	487	265	101	73	109
6.1	60	447	294	92	55	132
6.7	52	460	259	95	49	113
7.3	44	444	347	92	40	156
7.5	39	346	312	74	28	180
8.0	26	411	354	85	22	172
8.3	24	394	389	81	20	197
8.4	20	400	349	83	17	174
8.5	19	367	426	76	14	232
8.6	19	356	343	73	14	193
8.7	17	341	412	70	12	242
8.8	17	423	416	87	15	197
8.9	16	418	352	86	14	222
9.0	13	317	335	65	9	211
9.1	12	415	423	86	10	204

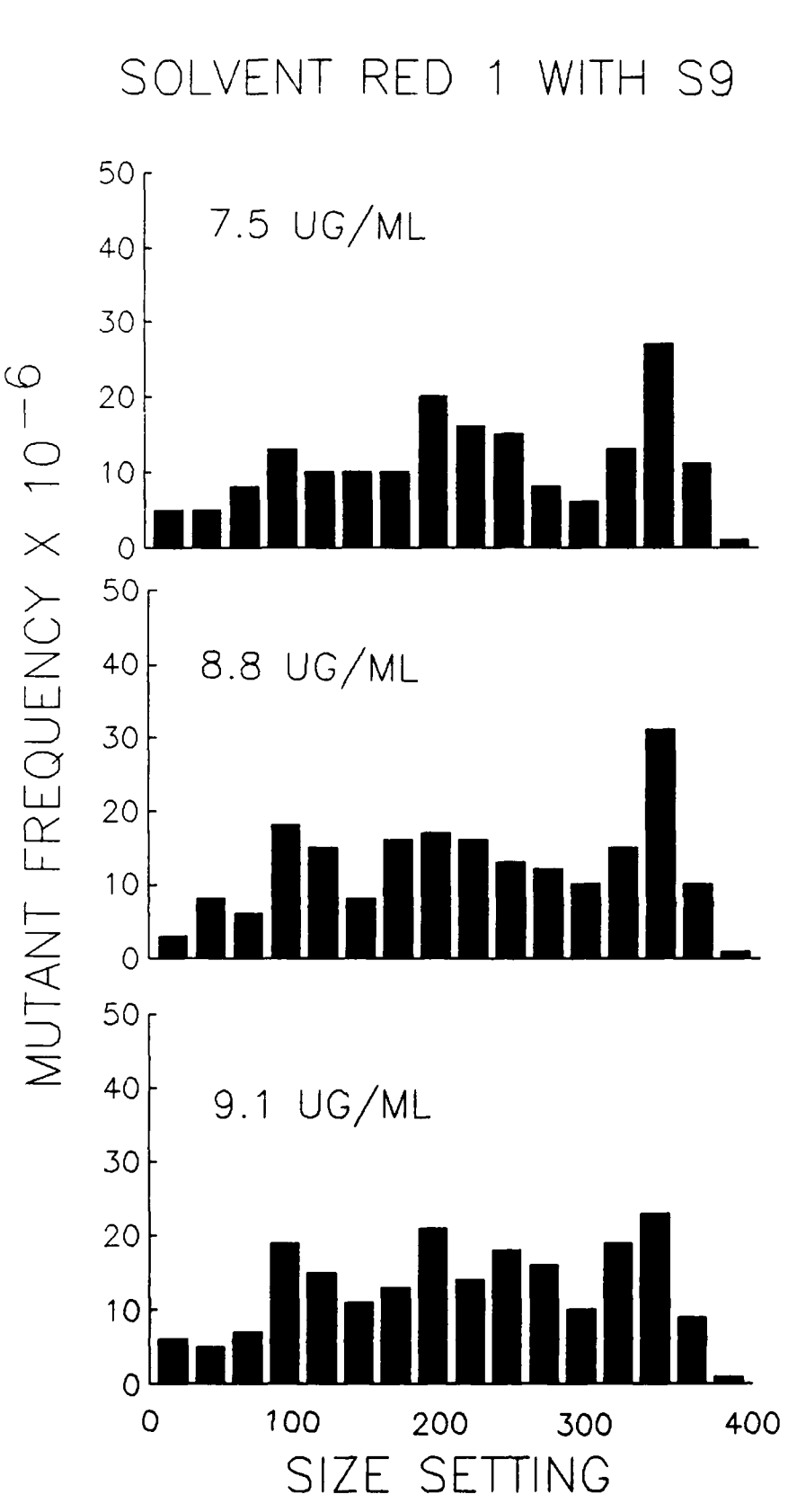

Figure 1. Colony sizing for TFT-resistant mutants following Solvent Red 1 treatment of mouse lymphoma cells with S9 metabolic activation. The small colonies are represented on the left side of size setting 250 on the histogram. The large colonies are to the right of size setting 250.

TABLE 8. MOUSE LYMPHOMA ASSAY OF DISPERSE BLUE 3 WITHOUT METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($\times 10^6$)
Experiment 1						
Neg. Control	100	498	110	100	100	44
Neg. Control	100	445	137	100	100	62
Pos. Control (EMS 400 μg/ml)	64	288	1848	61	39	1283
15	74	461	246	98	72	107
45	47	416	345	88	42	166
50	40	452	372	96	39	165
55	46	441	293	94	43	133
60	45	339	301	110	50	116
65	37	495	305	105	39	123
70	38	401	291	85	32	145
75	27	428	350	91	25	164
Experiment 2						
Neg. Control	100	460	196	100	100	85
Neg. Control	100	438	148	100	100	68
Pos. Control (EMS 400 μg/ml)	72	365	1556	72	59	853
15	87	442	307	97	85	139
30	63	500	371	110	70	148
60	46	444	364	98	45	164
70	35	392	395	86	31	202
80	36	383	461	84	31	241
90	39	353	474	79	31	269
100	24	282	479	63	15	340

and media were stained dark blue post-treatment. Cell pellets remained stained after rinsing with fresh media at all doses greater than 5 μg/ml.

S9 activation (Table 9) increased the magnitude of the mutant frequency induced by Disperse Blue 3 treatment. Once again, the compound stained cell pellets and media dark blue. Microscopic examination of supernatant obtained from day 1 dilutions indicated the presence of a chemical precipitate in both experiments at doses of 60 μg/ml or higher. These cultures were discarded, and results were not obtained for doses greater than 55 μg/ml. An examination of the colony sizing curves indicates that Disperse Blue 3 induced both small- and large-colony mutants with small-colony mutants predominating (Figure 2). This indicates that Disperse Blue 3 is likely acting by a clastogenic mechanism (Moore et al., 1985; Doerr et al., 1989).

Disperse Red 11 was clearly mutagenic without S9 activation at all doses ≥ 5 μg/ml (Table 10). The chemical went into solution in DMSO at 75 mg/ml. Post-treatment observation of the first experiment indicated that cell pellets were dyed red at all doses of 5 μg/ml or higher. By microscopic analysis, these cells appeared swollen, and centrifuge tubes were stained at all doses ≥ 15 μg/ml. Cell debris remained present on day 1 at 20 and 25 μg/ml. On day 1 of the second experiment, cells dosed at 25 μg/ml or higher remained dyed red. Microscopic observation indicated no crystals were present.

Disperse Red 11 was also positive with exogenous activation (Table 11). Supernatant from the wash and day 1 dilutions of both experiments were examined under the microscope for chemical precipitate. In the first experiment, large amounts of debris were present at all doses greater than 45 μg/ml. As a result, these points were discarded. Although debris was present in the second experiment and cells remained stained at doses of 40 μg/ml and greater, the debris were present in smaller quantities, appeared to consist of dead cells, and no crystals were observed. Disperse Red 11 induced both small- and large-colony mutants, indicating no preference for single gene or chromosomal mutations (Figure 3).

TABLE 9. MOUSE LYMPHOMA ASSAY OF DISPERSE BLUE 3 WITH METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($x10^6$)
Experiment 1						
Neg. Control	100	482	164	100	100	68
Neg. Control	100	518	205	100	100	79
Pos. Control						
(BAP 3 μg/ml)	86	488	645	98	84	264
(BAP 4 μg/ml)	51	491	1010	98	50	411
1	97	525	227	105	101	87
5	92	528	430	106	97	163
10	84	444	550	89	74	248
15	84	491	631	98	82	257
30	68	395	852	79	54	431
40	51	369	901	74	38	488
45	39	351	858	70	27	489
50	31	320	809	64	19	506
55	23	282	857	56	13	608
Experiment 2						
Neg. Control	100	496	180	100	100	73
Neg. Control	100	537	224	100	100	83
Pos. Control						
(BAP 3 μg/ml)	83	423	677	82	68	320
(BAP 4 μg/ml)	63	457	929	88	55	407
5	78	487	364	94	74	149
15	79	491	678	95	75	276
30	62	316	813	61	38	515
45	45	276	1070	53	24	575

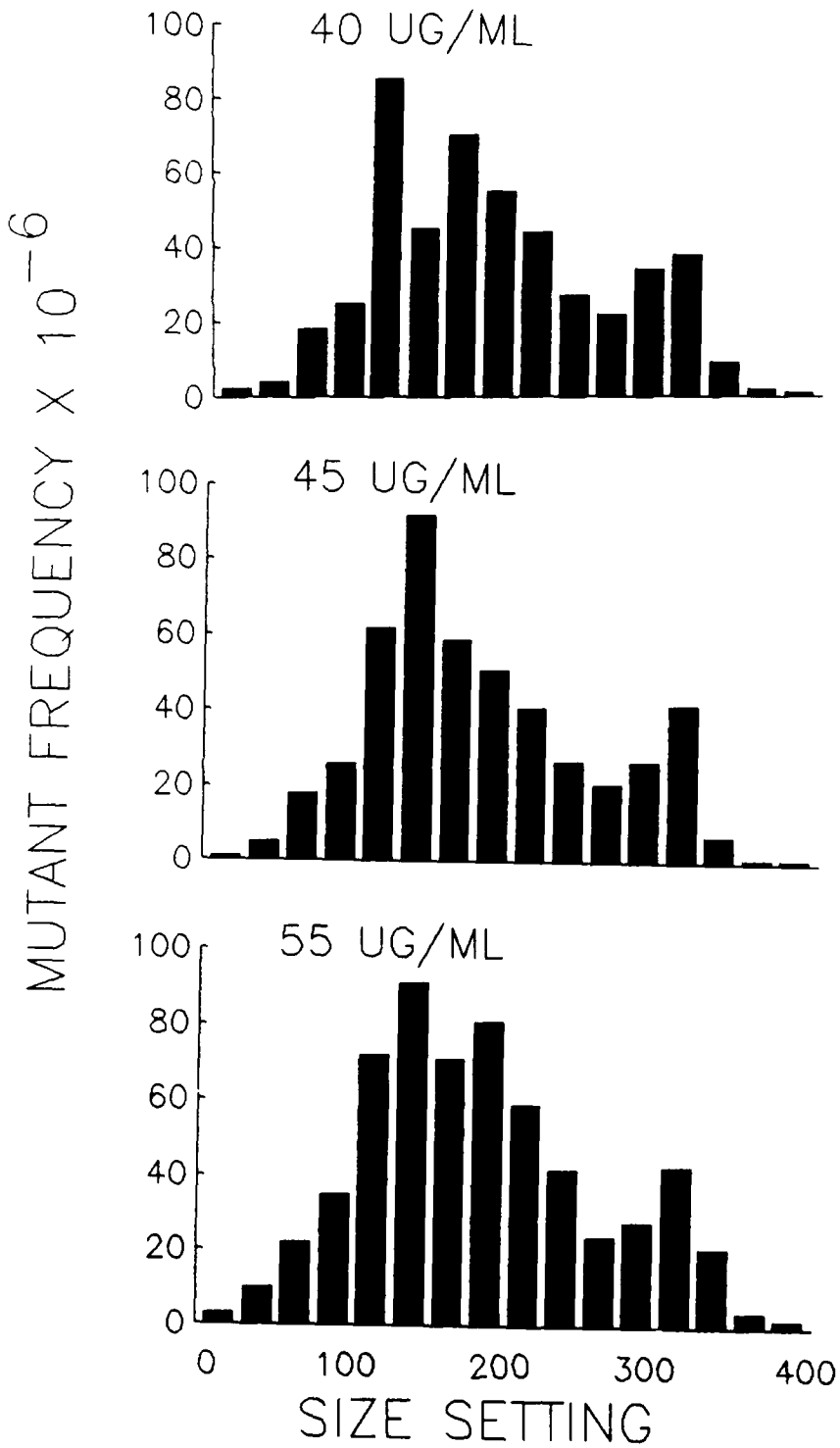

Figure 2. Colony sizing for TFT-resistant mutants following Disperse Blue 3 treatment of mouse lymphoma cells with S9 metabolic activation. The small colonies are represented on the left side of size setting 250 on the histogram. The large colonies are to the right of size setting 250.

TABLE 10. MOUSE LYMPHOMA ASSAY OF DISPERSE RED 11 WITHOUT METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($x10^6$)
Experiment 1						
Neg. Control	100	498	110	100	100	44
Neg. Control	100	445	137	100	100	62
Pos. Control (EMS 400 μg/ml)	64	288	1848	61	39	1283
5	87	338	366	72	62	217
10	82	372	340	79	64	183
15	67	389	398	82	55	205
20	65	335	356	71	46	212
25	69	348	315	74	51	181
Experiment 2						
Neg. Control	100	460	196	100	100	85
Neg. Control	100	438	148	100	100	68
Pos. Control (EMS 400 μg/ml)	72	365	1556	81	59	853
1	86	463	159	103	89	69
5	64	349	489	78	50	280
10	59	328	641	73	43	390
15	73	382	344	85	62	179
20	72	378	399	84	60	211
25	72	340	470	76	54	276
30	71	386	436	85	61	226
35	71	377	446	83	59	237
40	68	356	429	59	41	241

TABLE 11. MOUSE LYMPHOMA ASSAY OF DISPERSE RED 11 WITH METABOLIC ACTIVATION

Concentration (μg/ml)	Relative Suspension Growth (%)	Total viable Clones	Total Mutant Clones	Relative Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Freq ($\times 10^6$)
Experiment 1						
Neg. Control	100	496	180	100	100	73
Neg. Control	100	537	224	100	100	83
Pos. Control						
(BAP 3 μg/ml)	83	423	677	82	68	320
(BAP 4 μg/ml)	63	457	929	88	55	407
5	78	527	309	102	80	117
15	75	491	369	95	71	150
30	69	418	391	81	56	187
45	55	345	491	67	37	285
Experiment 2						
Neg. Control	100	588	233	100	100	79
Neg. Control	100	517	207	100	100	80
Pos. Control						
(BAP 3 μg/ml)	66	280	788	51	34	563
(BAP 4 μg/ml)	23	226	1204	41	9	1065
1	101	468	205	85	85	88
5	94	485	314	88	82	129
15	87	504	371	91	80	147
25	85	504	370	91	78	147
30	78	516	469	93	73	182
35	85	471	439	85	72	186
40	79	496	406	90	71	164
45	84	489	392	88	74	160
50	76	444	493	80	61	222
55	71	502	445	91	65	177
60	66	345	515	62	41	299
65	69	442	493	80	55	223

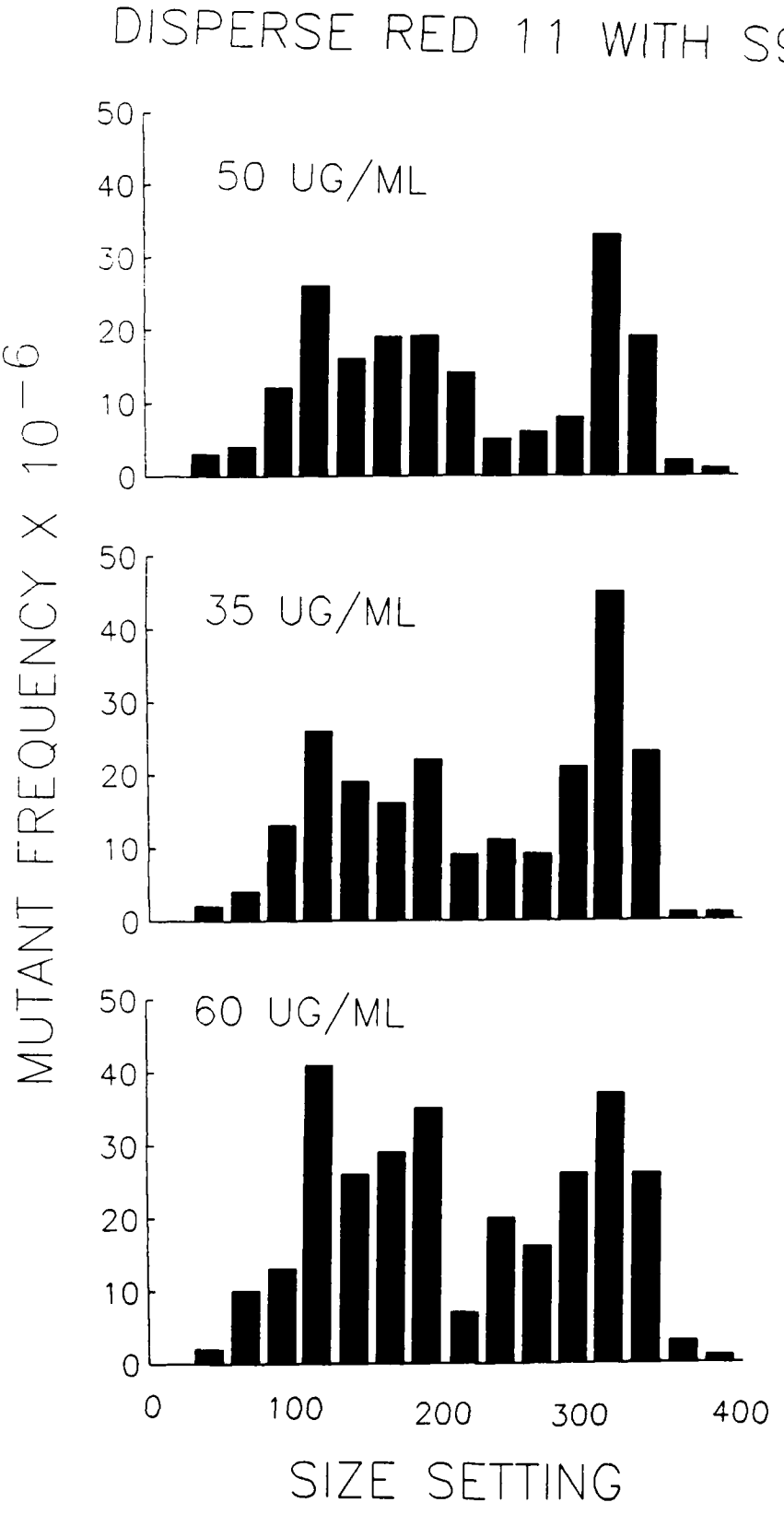

Figure 3. Colony sizing for TFT-resistant mutants following Disperse Red 11 treatment of mouse lymphoma cells with S9 metabolic activation. The small colonies are represented on the left side of size setting 225 on the histogram. The large colonies are to the right of size setting 225.

CONCLUSIONS

All three of the dyes tested gave a positive response to one of the six Ames tester strains. These positive responses were only observed in the presence of metabolic activation. The positive responses in the bacterial test system indicate that these compounds are capable of inducing point mutations. This mutagenicity was confirmed by the mouse lymphoma assay data. Because of its insolubility, Solvent Red 1 could not be tested without metabolic activation in the mouse lymphoma assay, but was testable and was weakly positive with activation. Colony sizing analysis indicated an induction of both single gene and chromosomal mutations. Disperse Blue 3 was more mutagenic when tested with rather than without S9 activation. Many of these mutants appeared to result because of the clastogenicity of Disperse Blue 3. Disperse Blue 3 is structurally similar to another anthraquinone dye, Disperse Blue 7, that is a potent mutagen to mouse lymphoma cells (Harrington-Brock et al., in press). The genotoxicity of Disperse Red 11 was approximately the same both with and without S9. Colony sizing indicated that both single gene and chromosomal mutations were induced.

In summary, all three of the tested dyes have the ability to induce mutations both in bacteria and in mammalian cells.

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